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Genetic Control of Virus Production in Cell Lines

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Summary

A procedure to maintain genetic control of virus production in tissue cell lines has been proposed and discussed. Both the tissue cell which replicates the virus and the virus inoculum must be homogeneous in order to produce a product with the expected characteristics. Certain philosophical and technical aspects of the problem are discussed in relation to developing and maintaining genetically homogeneous stock cultures, inoculum, and product.

Evidence from molecular genetics shows that variation of cell lines and viruses is similar to that of bacteria, fruit flies, and maize, for example, in that it is attributable to either changes in deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) for RNA-type viruses or to changes in the environment which allow previously unobserved phenotypic expression.[1,2] Variation in genotype or environment of either the cells or the virus may result in an altered interaction which affects the virus progeny qualitatively or quantitatively. The end product is the result of two biological systems and it is necessary, therefore, to maintain genetic and physicochemical control over both systems, the cell and the virus, in order to maintain uniformity of the process and thus the product.

This paper describes methods for controlling the production of a virus which uses cell lines as a host, as well as tests which may be employed to ascertain or assure the quality of the products. From the system described, one may derive principles useful for the control and production of viruses in other systems.

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PRESENT SITUATION WITH MAMMALIAN CELL STOCKS

Although there are exceptions, the present quality standards for stock cultures mammalian cells are generally low. For example, many cells lines used in published studies contain latent or inapparent viruses,[3] PPLO,[4] and bacterial contamination.[5] When antibiotics are used in the growth medium, contaminants are contrôlled to some degree, but in most cases become evident when growth is attempted in antibiotic-free medium. The presence of latent viruses may not be apparent unless the environment is changed or the cellular material is examined by electron microscopy.

Some cells lines are no longer correctly identified as to the species source. Presently, most cytogenetic studies are concerned with two characteristics: chromosome number and morphology. However, very few if any, mammalian cell lines can be characterized by a single test. Furthermore, few lines have been cloned and characterized for genetic homogeneity. Frequently, cell lines are subcultured continually on an irregular schedule using nonstandardized growth media, thereby allowing genetic variation to originate and to be retained in the culture.

PRESENT SITUATION WITH VIRAL STOCKS

Quite probably there is no genetically homogeneous viral stock in use today. In deriving viral stocks one must depend upon the physiological system of the host for replication of the virus. The biological variations in such systems may, therefore, result in heterogeneity in the viral progeny. In the case of a chick fibroblast system, for example, cells of unknown origin from all three germinal layers are present. These cells possess different enzymatic and physiological capabilities and are in various stages of differentiation. As a result, the virus produced may reflect the heterogeneity of the cells in which it is replicated.

Currently, with a few possible exceptions, we do not know how many particles constitute an infectious unit. We do not know that with some viruses, complete and incomplete viral particles occur. Since in phage-bacterial systems defective viral units may complement each other and further, since viral recombination can occur, there is a biological basis for assuming that these phenomena may occur with

mammalian viruses. There are, in fact, some reports of these phenomena.[6,7]

Plaque size is commonly accepted as a genetic marker for certain viruses. Our experience with VEE (unpublished) showed that we were unable to stabilize plaque size. This observation appears to be one common to other workers[8] and not unexpected since plaque size is dependent on several interacting factors.[9] Although other parameters such as antigenic differences, thermostability, and urea sensitivity have been used as genetic markers, we do not feel that these characters are discriminatively useful for selection for the character cannot be maintained, a case in point being the T markers of polio virus. In any case, the standards for homogeneity should approach those used by other microbiologists.

To minimize the foregoing deficiencies and difficulties the following basic procedures are proposed.

OUTLINE OF BASIC PROCEDURES

As indicated in Figure 1, from the selected strain of both the cell line and the viral stock, a single viable unit (i.e., a single cell of the cell line or plaque-forming unit of the virus) is obtained and grown to the quantity necessary for preparation of a stock culture. The possibility of selecting genetic mutants during the build-up is minimized by maintaining logarithmic growth of the cells under optimal conditions for the desired clone. This lessens selective pressures that might allow variants to proliferate and thus be selected.

For stabilization and preservation a procedure is employed which results in high initial recovery and maintenance of viability through the period of anticipated use, e.g., 5–25 yr. The suitability of the clonal stock is proven and a quantity of it is set aside as the proven primary stock culture which may be used at designated intervals to produce a secondary stock which, in turn, will produce the required inoculum. By returning to the proven primary stock, the possibility of uncontrolled genetic changes is minimized. Each secondary seed stock also is tested for its ability to produce the desired product used for a designated time, then replaced with another secondary inoculum derived directly from the primary seed stock. These successive steps are designed to maintain the homogeneity of the primary stock while a product is being produced. It should be noted that accept-

ance of an inoculum is based on two discriminating tests; one before acceptance of the stock culture as a proven one, the second on acceptance of the secondary stock. A third optional test also may be made on the inoculum itself.

Primary stock culture	Secondary stock culture	Inoculum
Selected strain		
↓		
Single viable unit		
↓		
Build-up		
↓		
Concentration		
↓		
Stabilization and preservation		
↓		
Testing and proving		
↓		
Acceptance		
↓		
Storage →	Mass transfer	
	↓	
	Build-up	
	↓	
	Stabilization or maintenance	
	↓	
	Proving tests	
	↓	
	Acceptance	
	↓	
	Storage →	Mass transfer
		↓
		Inoculum build-up
		↓
		Tests optional
		↓
		Use as inoculum or product
		↓
		Tests

Fig. 1. General scheme for seed stock maintenance and use.

PROCEDURE OF CHOICE AND DISCUSSION OF ITEMS

Desirable Standards

Since personal experience indicated that the quality of an operation deteriorates with use and time, a laboratory must deliberately set high standards of operation and quality assurance. For example, the risk of contamination and overgrowth of one cell line by another can be virtually eliminated by using only one cell line in a laboratory. Even with this restriction, our laboratory conducts fluorescent antibody tests at monthly intervals to document the species purity of cell lines. In addition, tests are made monthly to prove freedom from contamination with PPLO, bacteria, and molds. If tests are positive for contaminants, all material is destroyed and new inoculum is prepared from a pure primary or secondary stock culture, as the case may be. No antibiotics are used because of increasing the probability of masking PPLO and other contaminants. Use of laminar flow cabinets and/or white rooms are, in our experience, desirable for tissue culture production where antibiotics are not used in the growth medium. Many workers as well as ourselves have proven that antibiotic-free cell lines can be continued indefinitely without contamination.

Initial Stock Selection

In selecting a cell line, one must evaluate its efficiency in terms of viral spectrum, generation time, quality of cells and/or virus produced per milliliter of medium used, and medium complexity and cost. Some of these factors also can be used to screen clones.

Environmental Control

Growth environment is currently controlled by selection of the medium and continuous control of pH, oxidation–reduction potential, CO_2, and O_2. Instrumented New Brunswick fermentors or glass chambers of about 1 liter capacity are used for growth of the inoculum and final product.[10] Although there is still much to be learned about optimum physicochemical environments, it is apparent that genetic expression will be less subject to undesirable selective pressures in a controlled environment.

Cloning and Establishment of a Stock

All stocks and inocula are initiated from single-cell clones. A plating efficiency of at least 50% is desirable to minimize the possibility of genetic selection. For the L-M cell it is necessary to use a medium containing 20% (v/v) sterile, filtered horse serum to obtain a plating efficiency above ca. 30% (Carter et al., unpublished). The locations of single cells are marked after observation with a microscope, then the clones are picked 7–10 days after plating while the colony is still increasing in size. The selected colony is placed in 1 ml of medium in a test tube and held in a CO_2 incubator for 5 days. The resulting cell growth is transferred to a flask (Falcon T-30) containing 5 ml of medium from which either suspension cultures or monolayer cultures (Falcon T-60 flasks) may be prepared. Stocks are frozen at the time genetic characterization is made to eliminate the possibility of changes occurring upon further culture.

Preservation of the Stock Culture

Cells are grown in suspension to a concentration $1.5–2.0 \times 10^6$ cells/ml, then centrifuged at 4°C. The packed cells are resuspended to one tenth of the original volume in medium containing 5% glycerol or dimethylsulfoxide (DMSO) pH 7.0. One ml portions of cell suspension are placed in 2 ml vials and frozen in liquid nitrogen at the rate of 1°C/min through the range 4 to −50°C, then plunged into a liquid nitrogen storage tank. In order to obtain high recovery, it is necessary to work rapidly and maintain refrigerated conditions (4°C) from the time centrifugation is started until the cells are frozen.

Reinitiation of Growth

One vial of frozen stock is thawed and the cells transferred to 100 ml of medium in a 250 ml flask which is placed on a reciprocating or rotary shaker. Gaseous balance is maintained by rubber stoppers for 24 hr, after which some diffusion through a needle is allowed. The method and equipment have been described by which cultures can be grown uniformly and repeatably to concentrations of $1.5–2.0 \times 10^6$ cells/ml in 100 ml cultures in 7 days from the frozen stock.[11–13]

Proving of Primary Stock Cultures

At least 200 vials of stabilized and preserved stock are produced from each clone. Usually five tubes are used in tests to determine if

Reconstitute 5 tubes/clone
Transfer to 100 ml medium in 250 ml flasks
Agitate. Grow to 1.5–2.0 × 10^6 cells/ml
Inoculate new growth flask with 500,000 cells/ml
Serially transfer three more times

Tests:

- Doubling time for each 24 hr and overall
- Viable cell count
- Contamination: other cell lines, bacteria, PPLO and virus
- Genetic variation
  - Colonal type
  - Alkaline phosphatase
  - Zymogram analysis
- Virus production
- Other discriminatory tests

Standards:

Standards will need to be established which are specific for each clone in the growth environment being used. Standards should guarantee maintenance of genetic homogenity and high quality of product.

Destroy stocks not meeting all specifications.
Identify and accept clonal stock as primary stock culture.

Fig. 2. Acceptance tests and standards for the primary stock culture.

the quality of the stock is acceptable. The acceptance tests are outlined in Figure 2.

Preparation of Secondary Stock Cultures

A single vial of proven, primary stock culture is used to prepare a secondary stock from which the individual inocula used during a 6 month period are prepared. The culture is reconstituted, grown to the volume required, and preserved by procedures used with the primary stock culture. The same tests used for the primary are used to prove the secondary stock culture; however, specifications for primary and the secondary stock culture may be different. One vial of proven, secondary stock is used to prepare each process run. A lead time is required to prepare secondary seed and to conduct the quality assurance tests. The time needed will depend upon the tests conducted, since immunizing and/or virulence tests on a virus product may require an extended period. The use of a mass trans-

fer, rather than cloning, varies from our process with bacteria in which clones are used at both the secondary and inoculum steps[14] but is employed because of the long generation time of mammalian cells.

Viral Stocks

Currently, it is technically impossible to select and maintain a generally homogeneous virus stock. That any procedure will produce a clone originating from a single viral particle cannot reasonably be proven; moreover, the morphological and biochemical markers available today have little, if any, genetic meaning or discriminatory value. Nevertheless, it is necessary to attempt to minimize viral variation by following certain procedures.

(*a*) Select and use single-plaque isolates.

(*b*) Propagate virus to the required titer and volume in a cell system such as that just described by a series of transfers, rather than by obtaining virus from a culture which is produced over a long period or by repeatedly harvesting virus from the same culture. This procedure, we believe, decreases the chance of selecting for a less virulent variant from a virulent stock[15] since avirulent variants may not lyse the cell, but rather may be released continuously.[16]

(*c*) Clarify, stabilize, and preserve by procedures which maintain high viability. If the virus is to be preserved frozen, only 10% (v/v) skim milk need be added after clarification. However, if the virus is to be preserved as a lyophilized stock, then the stabilizer must be more specifically tailored to be specific requirements of each virus.

(*d*) Compare virulence, immunogenicity, or other cogent characteristic by various routes of inoculation in several hosts.

To reiterate, we are working without the required knowledge of viral genetics. The procedures employed were developed on the premise that viral inheritance is essentially like that of other organisms and that the use of these procedures will "minimize" variation.

FUTURE WORK

Better control of the genetics of mammalian cells will be gained when isoenzyme and other enzyme systems are more fully understood. From preliminary work in our laboratory, using selected isoenzymes, it

appears possible to distinguish between and among the L-M and L-DR lines as well as a number of clones of these lines as shown in Table I (unpublished work from this laboratory). No single enzyme is a suitable marker for all the clones; however by using a combination of isoenzymes, different patterns emerge for each clone. Further development of this method will allow not only identification of each stock, but also detection of genetic change in a stock. Since at present the significance of the Type A, B, and C particles found in some L cultures has not been evaluated, a record of these particles should be kept on all cell stocks. For viral stocks, techniques must be developed which will allow isolation of viral mutants independent of living systems. Selection based on: (*1*) altered base composition of the viral DNA and/or RNA, (*2*) use of altered electrical charges of the viral particles which reflect changes in amino acid content, and (*3*) alteration of the active attachment site of the capsomer are promising methods of approach, however, due to the pace of molecular biology other approaches will soon become attractive.

TABLE I
Hypothetical Distribution of Enzymes in Cell Lines

	Cell line			
Enzyme quantitated	L-M	L-DR	Clone 1-2 of L-Ma	Clone 1-7 of L-Ma
Isoenzyme 1 Bands	A		A	A
A, B, and C	B	B	B	
	C	C	C	C
Isoenzyme 2 Bands,	A	A	A	No bands
A and B		B		
Isoenzyme 3 Bands	A	A	A	A
A, B, C, and D	B	B		B
	C	C	C	
	D	D	D	D

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